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Problems in the Interpretation of HIV-1 Viral Load Assays Using Commercial Reagents

Siobhan O'Shea,[1]* Ian Chrystie,[1] Ross Cranston,[2] Jane Mullen,[1] Karen Corbett,[1] Gary Murphy,[3] John V. Parry,[3] Annemiek De Ruiter,[2] and Jangu Banatvala[1]

[1]Department of Virology, Guy's and St. Thomas' Hospital Trust, London, United Kingdom
[2]Department of Genito-Urinary Medicine, Guy's and St. Thomas' Hospital Trust, London, United Kingdom
[3]Hepatitis and Retrovirus Laboratory, PHLS Virus Reference Division, Central Public Health Laboratory, Colindale, London, United Kingdom

During routine monitoring of human immunodeficiency virus (HIV) viral load, two problems arose. First, a number of patients, the majority being African, were found to have low viral loads by the Chiron branched-chain DNA assay in conjunction with low $CD4^+$ cell numbers. In order to determine whether this was due to failure of the branched-chain DNA assay to detect non-B subtypes of HIV, selected samples were subtyped and HIV RNA quantified by branched-chain DNA, NASBA, and the Roche Monitor RT-PCR assay. Twenty-eight (97%) of 29 Africans were infected with a non-B subtype of HIV and 15 (93.7%) of 16 non-Africans with subtype B. Twenty-three samples had a low viral load by branched-chain DNA, which was confirmed by the NASBA and RT-PCR assays. All three assays detected B and non-B subtypes with similar efficiency; NASBA failed to detect HIV RNA in a small number of non-B samples. Discrepancies between viral load and $CD4^+$ cell numbers did not appear therefore to be related to subtype. Second, while quantification of HIV RNA was being conducted using version 2 of the branched-chain DNA assay (lower detection limit 500 HIV RNA copies/ml) the manufacturers had developed a more sensitive assay and a comparative evaluation was therefore conducted. In approximately 30% of samples the viral load was up to 10 times higher with the more sensitive assay. These experiences emphasise the importance of close collaboration between the clinic and the laboratory. ***J. Med. Virol. 61:187–194, 2000***.

KEY WORDS: HIV-1; RNA quantification; HIV-1 subtypes

INTRODUCTION

Three commercial assays are currently available for the quantification of human immunodeficiency virus type 1 (HIV-1) RNA in plasma (branched-chain DNA assay (bDNA), Chiron Diagnostics; Amplicor HIV-1 Monitor assay, (RT-PCR) Roche Diagnostic Systems; nucleic acid sequence-based amplification (NASBA), Organon Teknika). Their main application is in the initiation and monitoring of anti-retroviral therapy. All three were initially developed using the B subtype of HIV-1, which predominates throughout Europe and North America, and their efficiency in detecting non-B subtypes is variable [Coste et al., 1996; Alaeus et al., 1997; Gobbers et al., 1997; Damond et al., 1999; Parekh et al., 1999]. Indeed, the initial version of the Roche Monitor assay (version 1.0) failed to detect a high proportion of non-B subtypes [Coste et al, 1996; Alaeus et al., 1997; Triques et al., 1999] until non-B primers were added to the assay (version 1.5). The three commercial viral load assays continue to be developed and modified by the manufacturers and more sensitive assays are now available. As quantitative assays evolve, and new assays become available, close collaboration between the laboratory and the clinic is essential to assess the impact of this on patient management.

Routine monitoring of HIV viral load has been conducted over a 2-year period among patients attending Genito-Urinary Medicine (GUM) clinics at Guy's and St. Thomas' Hospital Trust using the Chiron bDNA assay. During this time, anecdotal reports from routine clinical practice suggested discrepancies between viral load and $CD4^+$ cell numbers in some patients, the majority of whom were African. These patients, who were not being treated with antiretroviral drugs, had low or undetectable viral loads in conjunction with low (<300 cells/cu mm) $CD4^+$ cell numbers. This made it difficult to decide whether to start antiretroviral therapy and posed problems for subsequent monitoring of treatment. The question was raised as to whether the viral

*Correspondence to: Siobhan O'Shea, Department of Virology, Guy's and St. Thomas' Hospital, Trust, Lambeth Palace Road, London SE1 7EH, United Kingdom.
E-mail: siobhan.o'shea@gstt.sthames.nhs.uk

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TABLE I. Details of Three Commercial Assays for Quantification of HIV-1 RNA

Assay	Details	HIV target region	Sample volume	Dynamic range RNA copies/ml
Amplicor Monitor version 1.5	Competitive RT-PCR	Gag	200 μl	400–750,000
NASBA	Competitive isothermal enzymatic nucleic acid amplification	Gag	200 μl	400–1,000,000
bDNA version 2	Hybrid capture, signal amplification	Pol	2 ml	500–750,000
bDNA version 3	As for version 2	As for version 2	1 ml	50–500,000

load in these patients was genuinely low or represented failure of the bDNA assay to quantify non-B subtypes of HIV accurately. If this were the case, it would also result in failure to recognise the need for a change in treatment among individuals taking antiretroviral drugs. At the same time, a Public Health Laboratory Service anonymous survey had demonstrated that although subtype B was the major subtype among intravenous drug users (97%) and male homosexuals (99%) in London, non-B subtypes were more common among individuals infected heterosexually, particularly when infection was associated with the African continent (manuscript in preparation). Stored plasma samples, selected on the basis of the patients' ethnic origin and viral load (bDNA, version 2), were therefore subtyped and viral load determined additionally using the reverse transcription-polymerase chain reaction (RT-PCR) and NASBA assays. Also during this period, Chiron Diagnostics introduced a more sensitive bDNA assay (version 3) with a lower detection limit of 50 HIV RNA copies/ml. This compared with a lower detection limit of 500 copies/ml for version 2 of the assay, which was due to be withdrawn. Although the manufacturers stated that the viral load was only likely to be two- to threefold higher with the more sensitive assay, it was considered important to evaluate the significance of this in patients attending the HIV Unit at Guy's and St. Thomas' Hospital Trust, which currently cares for more than 850 regular attendees per year.

MATERIALS AND METHODS

Viral load assays

Three commercial HIV-1 viral load assays for quantification of HIV-1 RNA were used. Amplicor HIV-1 Monitor Assay Version 1.5 (RT-PCR), (Roche Diagnostic Systems), nucleic acid sequence-based amplification (NASBA) QT HIV-1 assay (Organon Teknika Ltd), and branched-chain DNA (bDNA), Quantiplex HIV-1 assay versions 2 and 3 (Chiron Corporation). Details of the assays are given in Table I, and all were conducted according to the manufacturers' instructions.

Efficiency of three viral load assays for detection of non-B subtypes of HIV

Study group

Stored plasma samples were tested from 55 HIV-positive individuals attending GUM clinics at Guy's and St. Thomas' Hospital Trust (Table II). Samples were selected on the basis of ethnic origin (African n = 35, non-African n = 20), $CD4^+$ cell number and viral load, which had already been determined routinely using version 2 of the bDNA assay. Thirty-nine of the 55 individuals were being treated with antiretroviral drugs (Table II). Samples were selected from Africans and non-Africans with apparent discrepancies between their viral load (<500 copies/ml) and $CD4^+$ cell number (<300 cells/mm^3) together with samples selected at random from Africans and non-Africans with similar viral loads (Table II). Viral load was also determined on stored samples from these 55 patients using the RT-PCR and NASBA assays.

HIV Subtyping

All 55 samples were initially subtyped using a peptide-based serotyping EIA as described previously [Murphy et al., 1999]. The serotyping assay is an indirect assay using synthetic oligopeptide antigens representing each HIV-1 subtype from A to E and HIV-1 O. The peptides were prepared from consensus sequences of each of the V3 loops. A serotype was assigned to a specimen on the basis of the oligopeptide with which greatest activity was observed. Although this assay uses synthetic oligopeptides from a number of HIV-1 subtypes, it can only be used reliably to distinguish subtype B from non-B subtypes. This is because antibodies raised against various non-B subtypes are cross-reactive [Murphy et al., 1999]. Where demographic data indicated that the subtype, as determined by serotyping, was unexpected (e.g., non-B subtype in a homosexual subject) genotypic analysis was conducted using a heteroduplex mobility assay (HMA) as described previously [Novitsky et al., 1996; Belda et al., 1997]. In brief, HMA was performed using equal amounts of amplified DNA from the sample (100–200 ng) and each reference plasmid, combined in 50 mM NaCl, 5 mM Tris, pH7.8, 1 mM EDTA, heated to 94°C for 2 min and incubated at 4°C for 10 min. Samples were mixed with 1.5 μl of 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol, and loaded on a 3% metaphor XR agarose horizontal gel containing 1 M urea. Electrophoresis was at 125 V for 3–4 hr in 1× TBE buffer and the homoduplexes and heteroduplexes detected by staining with ethidium bromide. The HMA kit was supplied by the NIH AIDS Research and Reference reagent Program through the UK MRC AIDS Reagent Project.

TABLE II. Samples Used to Evaluate the Efficiency of Three Commercial Viral Load Assays for Detection of Non-B Subtypes of HIV

Group	No.	No. being treated with ARV[a]	HIV copy no./ml Chiron bDNA	Median CD4 cell no. (range)
Non-African	9	8	<500	221 (64–405)
African	12	10	<500	247 (25–396)
African	2	1	<500	621 (560–682)
Non-African	6	2	500–10,000	390 (64–1008)
African	15	7	500–10,000	221 (10–980)
Non-African	5	5	>10,000	126 (30–360)
African	6	6	>10,000	140 (99–288)
Total:	55	39		

[a]ARV antiretroviral agents.

Comparative evaluation of bDNA assay versions 2 and 3

Study group

Stored plasma samples from 196 HIV-positive individuals attending GUM clinics at Guy's and St. Thomas's Hospital Trust, which had been tested routinely using version 2 of the bDNA assay, were tested in addition using the more sensitive version 3 assay. Sixty-two samples had a viral load of <500 copies/ml and 134 a viral load from 500 to >100,000 copies/ml using version 2 of the bDNA assay.

Statistical Analysis

HIV copy numbers were not distributed normally and were therefore $\log_{10}$-transformed for analysis. Comparative evaluation of versions 2 and 3 of the bDNA assay was conducted using methods described previously [Bland and Altman, 1986].

RESULTS

Efficiency of Three Viral Load Assays for Detection of Non-B Subtypes of HIV

Twenty-three of the 55 samples (14 African, 9 non-Africans) had undetectable viral loads by bDNA (version 2). Ten of these samples (43.5%) also had undetectable viral loads by the RT-PCR and NASBA assays and in the remaining 13 (56.5%) the HIV RNA copy number was low (range: 400–8,000 copies/ml, Fig, 1). The low viral loads originally detected were therefore confirmed by the two other assays. These results were not influenced by ethnicity, being apparent both with samples from Africans and non-Africans. Most African patients were infected with non-B subtypes of HIV (see below). Fifteen (8 African, 7 non-African) of the 23 samples with undetectable or low viral loads were from individuals with low $CD4^+$ cell numbers ($<300/mm^3$). Among the remaining 32 samples (Fig. 1), the median HIV RNA copy number was lower with the bDNA assay (3,064 copies/ml) than with the NASBA (16,000 copies/ml) and RT-PCR (29,733) assays, regardless of the individuals' ethnic origin.

All 55 samples were subsequently serotyped, and 13 (23.6%) were subtype B and 32 (58.1%) non-B; 8 (14.5%) of the remaining samples were multiply reactive, and 2 (3.6%) were below the detectable limit of the subtyping assay. These 10 samples were therefore not included in further subtype analyses, which were conducted only on the samples identified as B or non-B. Twenty eight of 29 (97%) patients of African ethnicity were infected with a non-B subtype of HIV and 12 of 16 (75%) non-African patients were infected with subtype B. Four non-Africans, three infected homosexually and one heterosexually, therefore appeared to be infected with a non-B subtype. These four specimens were serotyped again using a wider range of dilutions. Two of the three samples had weak reactivity to the subtype B peptide at a 1:50 dilution and were confirmed as subtype B by genotyping. One sample was again serotyped as non-B but genotyped as subtype B, and the remaining sample, although repeatably serotyped as non-B, could not be amplified for genotyping. It was concluded that three of the four samples were subtype B, while one was indeterminate and therefore not included in further subtype analyses. The differences between genotyping and serotyping results was attributed to a change from the consensus sequence of the V3 loop of gp120 of the infecting virus as described previously [Sherefa et al., 1997; Murphy et al., 1999].

Figure 2 shows the viral load, determined by the three assays, for 44 samples that were subtyped as B (n = 16, Fig. 2a) or non-B (n = 28, Fig. 2b). Although results by these assays differed in terms of absolute copy numbers, this did not appear to be related to subtype. Thus, although the median copy number was lower with the bDNA assay, all assays appeared to quantify B and non-B subtypes with similar efficiency (Fig. 2a,b). However, using NASBA, a small number of non-B subtypes were either not detected or gave substantially lower copy number. Of 28 non-B samples 4 (14%) had a copy number of 0.7 $\log_{10}$ or more lower with NASBA than with the RT-PCR assay, compared with only 1 of 16 (6%) subtype B samples.

Comparative Evaluation of bDNA Assay Versions 2 and 3

Among the 196 samples tested, 123 had a viral load within the dynamic range of both version 2 and version 3 of the bDNA assay. In Figure 3, the average $\log_{10}$ copy number for each of these 123 samples, as tested by the two assays, is plotted against the $\log_{10}$ difference and the mean difference in copy number, given together with the limits of agreement between the two

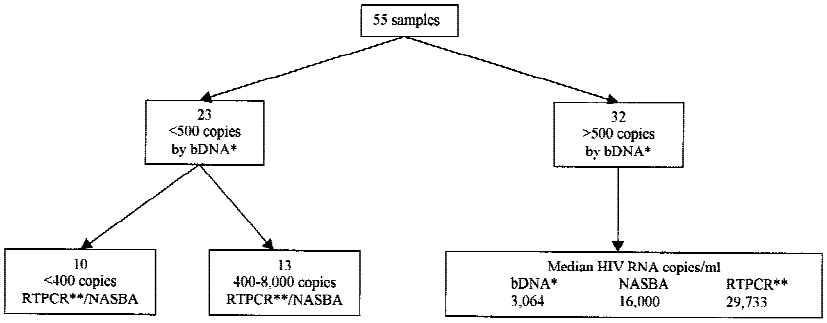

Fig. 1. Comparison of three commercial assays for quantification of human immunodeficiency virus (HIV) RNA.

assays as ±2 SD [Bland and Altman, 1986]. Overall, version 3 of the assay gave higher copy numbers than version 2, with a mean difference between the two assays of 0.4 $\log_{10}$ (confidence interval [CI] 0.37–0.45; limits of agreement 0.01–0.84 $\log_{10}$). Differences between the two assays tended to be more apparent at lower concentrations of HIV RNA. In 73% of samples, there was no more than a 0.5 $\log_{10}$ difference in copy number between the two assays, but in the remainder the difference was within the range of 0.52–1.2 $\log_{10}$.

Among the 73 samples outside the dynamic range of both assays, 62 had a viral load of <500 copies/ml by version 2 of the assay (Fig. 4). In 27 (43.5%) of these samples, the viral load was <50 copies/ml by the more sensitive assay, and in 35 (56%) the viral load was within the range of 55–5,139 copies/ml (Fig. 4). Eleven samples with viral loads of >500,000 by version 3 of the bDNA assay had copy numbers within the range of 208,000–566,040 by version 2 (data not shown).

DISCUSSION

During routine monitoring of HIV viral load, there was concern that a low viral load, in conjunction with a low $CD4^+$ cell number, in patients not being treated with antiretroviral drugs, might be due to the failure of the bDNA assay to detect non-B subtypes of HIV-1. However, evaluation of selected patient groups, demonstrated that in both African and non-African patients, low viral loads as measured by bDNA were confirmed by the RT-PCR and NASBA assays. Although a high prevalence of non-B subtypes was demonstrated among African patients, the discrepancy between viral load and $CD4^+$ cell number did not seem to be related to subtype, and the low and undetectable viral loads appeared to be accurate. Most samples evaluated were from individuals being treated with antiretroviral drugs and undetectable viral loads in conjunction with low $CD4^+$ cell numbers could be explained by lack of immune restoration despite a successful virological response [Hammer et al., 1996; Schooley et al., 1996; Pakker et al., 1997, 1999; Li et al., 1998]. However, what might account for such discrepancies in individuals who have not been treated with antiretroviral drugs remains unclear.

In a small proportion of non-B samples (~14%), the HIV RNA copy number, as determined by NASBA, was substantially lower or undetectable compared to results obtained with the other two assays. This phenomenon has also been observed during paediatric diagnosis of HIV (data not shown). In eight infected African infants and their mothers, although proviral DNA was detected by the Roche Amplicor assay, viral RNA was either not detected or was detected only at a low copy number by NASBA. When infant specimens were tested by the RT-PCR assay, however, HIV RNA was detected and at a higher concentration. The qualitative NASBA has been used to determine the HIV infection status of infants born to seropositive mothers since the sample volume from infants is generally too small for the bDNA assay (2 ml for version 2; 1 ml for version 3). However, as most HIV-positive women attending clinics are of African origin, and likely to be infected with a non-B subtype, the RT-PCR assay is now used for paediatric diagnosis. Preliminary evaluation of sample volumes of less than 1 ml in the bDNA assay have been conducted and reliable results can be obtained with 100-250μl of plasma. The bDNA assay may therefore be appropriate for paediatric diagnosis using this modification.

Failure of NASBA to detect HIV RNA in some African patients may also affect management of HIV infection during pregnancy. In accordance with CDC guidelines [CDC, 1998] HIV infected pregnant women with low viral loads and good $CD4^+$ cell counts are offered zidovudine monotherapy and elective caesarean section to reduce the risk of vertical transmission, whereas for women with high viral loads combination therapy is recommended. It has been demonstrated that in some cases although HIV viral load may be undetectable by NASBA, the RT-PCR assay subsequently reports a substantial HIV RNA copy number. The outcome could be an initial recommendation for monotherapy, which is subsequently changed to combination therapy. This could be particularly significant in the management of women who present late and very close to term, when antiretroviral therapy needs to be commenced without delay. Such results could also compromise the trust that the woman has in the clini-

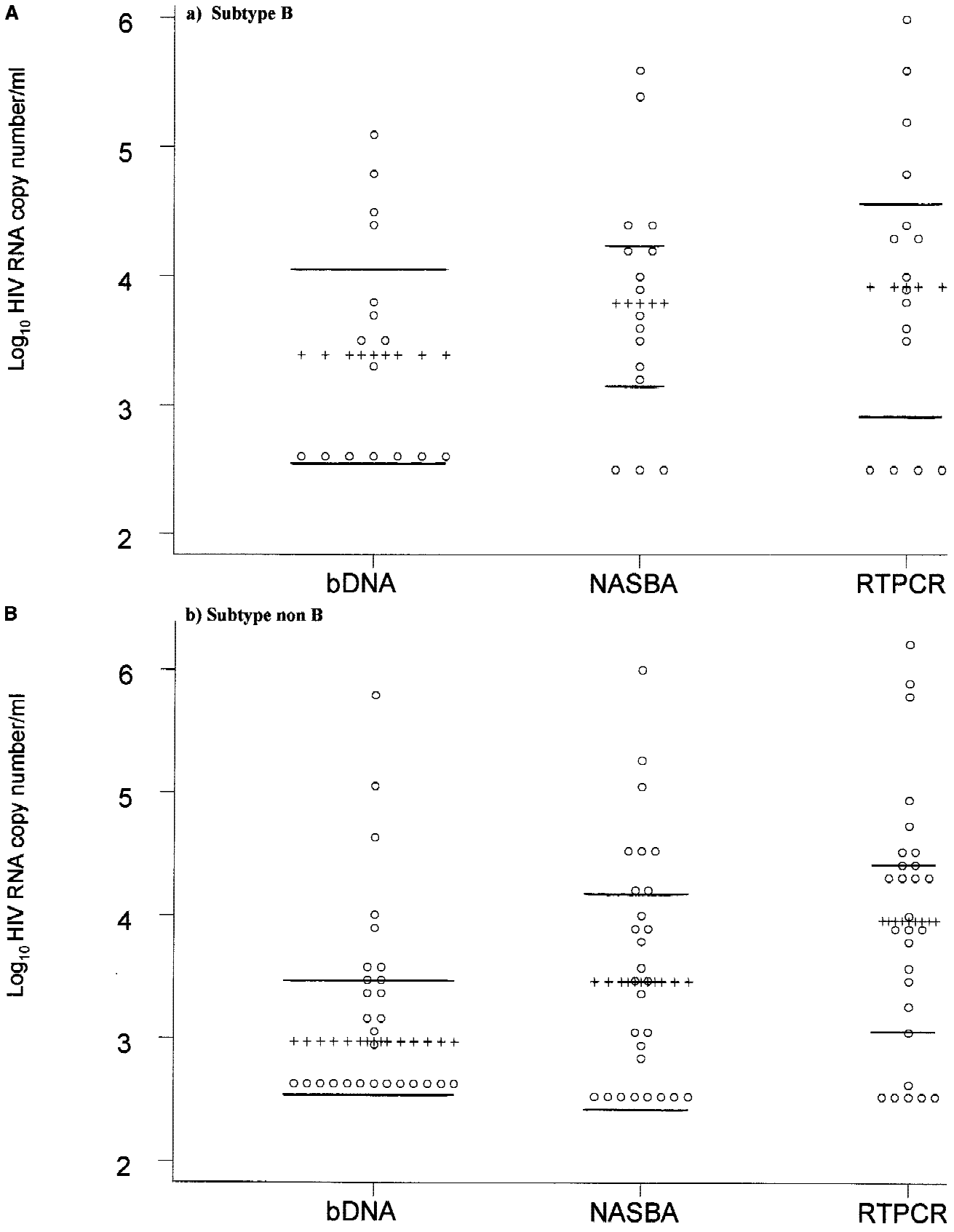

Fig. 2. Quantification of human immunodeficiency virus (HIV) RNA in 16 subtype B and 28 non-B subtypes, using branched-chain DNA (bDNA) (version 2) assay, nucleic acid sequence-based amplification (NASBA) assay, and reverse transcription-polymerase chain reaction (RT-PCR) (version 1.5) assays. ++++, median copy number; ___, 25th and 75th centiles.

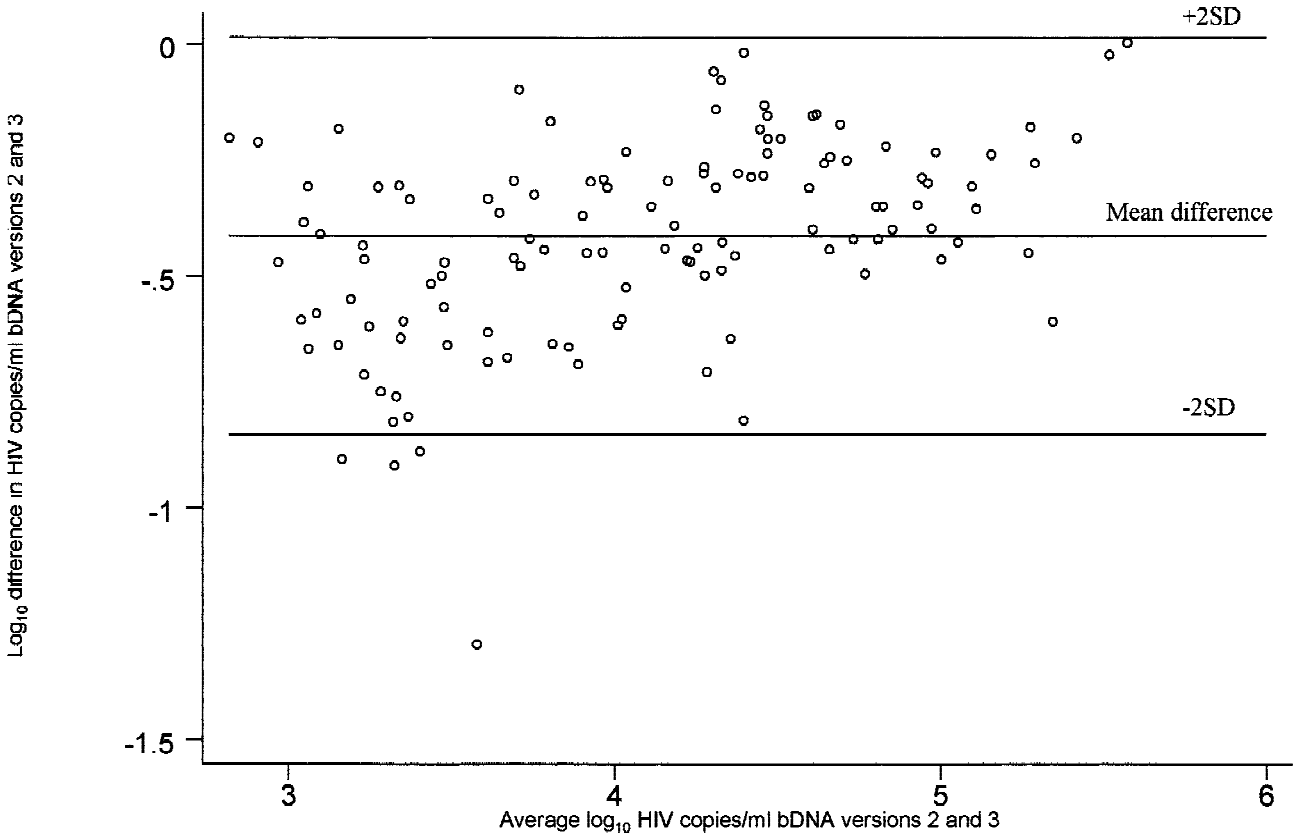

Fig. 3. Comparative evaluation of branched-chain DNA (bDNA) (versions 2 and 3) assay for quantification of human immunodeficiency virus (HIV) RNA.

cian who is guiding her through this crucial phase. Regular communication between the virologist and clinician will reveal these inconsistencies and help resolve them.

In common with others [Prud'homme et al., 1998; Segondy et al., 1998; Ginocchio et al., 1999], it has been demonstrated that version 2 of the bDNA assay gives lower absolute copy numbers than either the RT-PCR and NASBA assays. This can affect patient management as one of the criteria for initiating antiretroviral therapy may be a viral load of >10,000 copies [BHIVA, 1997]. Thus, among the 55 patients tested in this study using three viral load assays, 10 (18.1%) might have been eligible for therapy if tested by bDNA compared to 20 (36.3%) by NASBA and 29 (57.7%) by RT-PCR. More recent guidelines do, however, suggest a higher threshold for treatment [BHIVA, 1998]. The original data on which the threshold of 10,000 copies/ml for initiation of treatment was based were derived using the bDNA version 2 assay [Mellors et al., 1995, 1996] and, as demonstrated in this study, this threshold may not be appropriate to other HIV RNA assays. It is therefore contended that guidelines that specify a particular viral load as a criterion for treatment should also specify the assay to be used [Devereux et al., 1999], and that the same assay be used throughout patient monitoring.

During the course of our first 2 years of routine monitoring of HIV viral loads using the bDNA assay, the manufacturers introduced a more sensitive assay. Although the mean difference in viral loads between the two assays was 0.4 $\log_{10}$ (confirming the manufacturer's statement that values would be two- to threefold higher), in some cases the viral load was up to 10 times higher by version 3 of the assay. On the basis of this evaluation, in most cases the viral load would not be expected to be clinically significantly different using the more sensitive assay but in 20–30% of individuals the estimate might be significantly higher. A particular problem concerned those individuals who had a viral load of <500 copies with version 2 of the assay since 56% changed from what they perceived as undetectable to detectable with a viral load of 50–5,000 copies/ml. This evaluation enabled an estimate of the likely impact of the results obtained by the new assay once it was introduced into the clinic. For most patients, no change in therapy was indicated but in a small proportion a change in treatment was suggested. Some patients would also be disappointed to have a detectable viral load when it had been undetectable previously. In anticipation of these problems, virologists and clinicians involved in the treatment of HIV-infected patients met on a number of occasions. An information sheet was prepared that explained the change in assay, outlined the possible effects of results, and announced the date that the new assay would come in to use. These were given to all patients attending the unit for

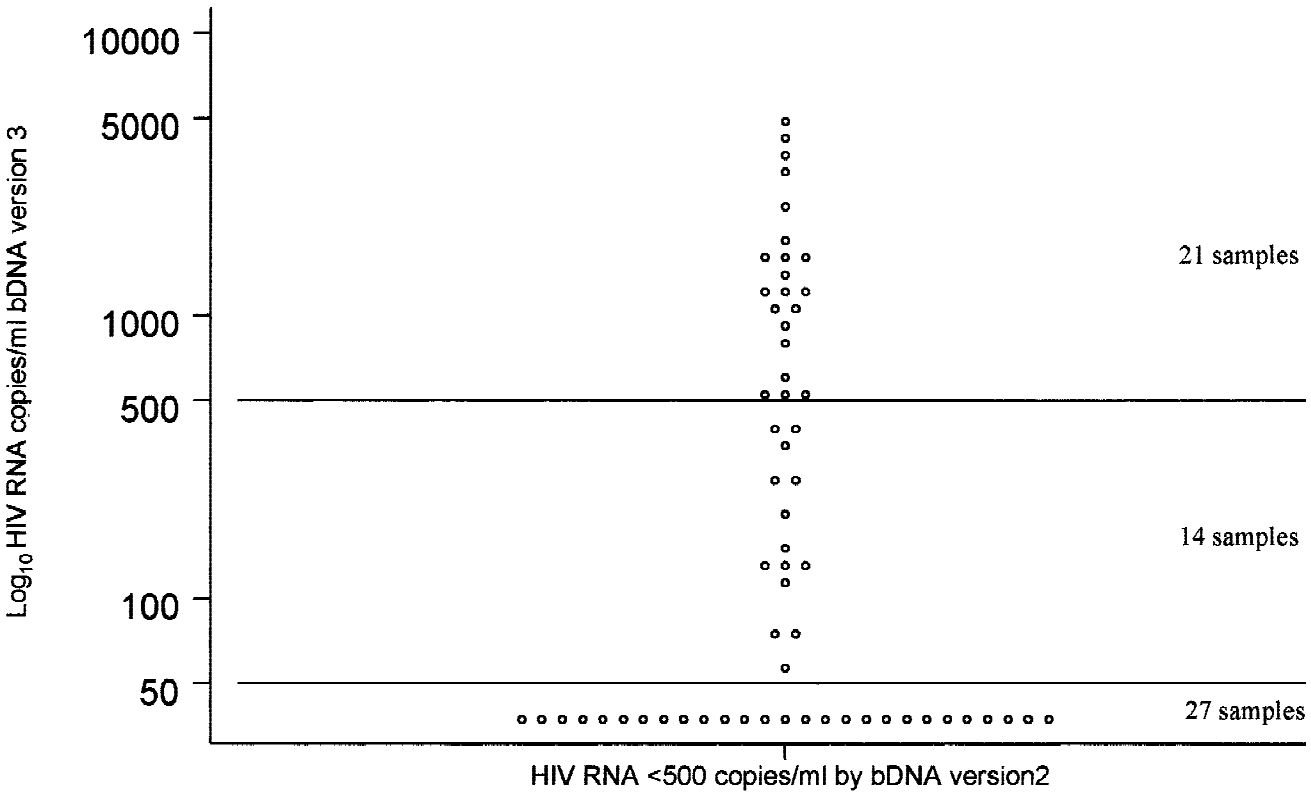

Fig. 4. Quantification of human immunodeficiency virus (HIV) RNA by branched-chain DNA (bDNA) (version 3) assay in 62 samples with a viral load of <500 copies/ml by bDNA (version 2).

4 weeks before the introduction of the new assay. Information was also disseminated to all doctors and nurses working in the clinic. In this way, patients and staff were prepared for possible changes in viral load results.

Experience with a routine HIV viral load service has demonstrated the importance of close liaison between the laboratory and clinic. These findings also emphasise the need for manufacturers to ensure better calibration between the methods in use and of high-quality consistent production. Moreover, the essential requirements of internal quality control and performance assessment must be in place in testing laboratories to ensure reliable and accurate quantification of HIV RNA.

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